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(54) Title: BIOLOGICAL REGULATION OF MINERALIZATION

(57) Abstract

An aspartic acid rich protein isolated from human urine, as well as proteins having substantial homology thereto and active portions of the foregoing are effective modulators of mineralization in mammals. These proteins and peptides are useful as therapeutic agents, such as in the treatment of kidney stone disease. Hybridoma cell lines capable of producing monoclonal antibodies to these proteins and peptides and monoclonal antibodies produced by these hybridomas are disclosed. These monoclonal antibodies are also useful as therapeutic agents, such as in the treatment of osteoporosis, and further have utility as diagnostic agents. Other uses are also described.

BIOLOGICAL REGULATION OF MINERALIZATION

REFERENCE TO GOVERNMENT GRANT

This work was supported in part by research grants from the National Institutes of Health, grant numbers DK-33501, DK-07006, DK-30280, and AR-20553. The United States Government may have certain rights in this invention.

BACKGROUND OF THE INVENTION

Urinary tract stone disease is a common human malady. The vast majority of stones formed in the urinary space are mineralized with calcium salts. Meyer et al., *Invest. Urol.*, Vol. 13, pp. 36-39 (1975); Coe et al., *Kidney Int.*, Vol. 38, pp. 625-631 (1990). Although normal urine is frequently supersaturated with respect to calcium oxalate, inhibitors are thought to protect most humans from the formation of stones. Nakagawa et al., *J. Biol. Chem.*, Vol. 258, pp. 12594-12600 (1983). The most abundant protein in normal urine, Tamm-Horsfall protein (TH), Tamm et al., *Proc. Soc. Exp. Biol. Med.*, Vol. 74, pp. 108-114 (1950), however, has been found to be inactive as an inhibitor of crystal growth, Worcester et al., *Am. J. Physiol.*, 255, F1197-F1205 (1988), and attempts to identify proteins or other factors that fill this role have hereto-fore been only partially successful.

Ile-Thr-Ser-His-Met-Glu-Ser-Glu-Glu-Leu-
Asn-Gly-Ala-Tyr-Lys-Ala-Ile-Pro-Val-Ala-
Gln-Asp-Leu-Asn-Ala-Pro-Ser-Asp-Trp-Asp-
Ser-Arg-Gly-Lys-Asp-Ser-Tyr-Glu-Thr-Ser-
Gln-Leu-Asp-Asp-Gln-Ser-Ala-Glu-Thr-His-
Ser-His-Lys-Gln-Ser-Arg-Leu-Tyr-Lys-Arg-
Lys-Ala-Asn-Asp-Glu-Ser-Asn-Glu-His-Ser-
Asp-Val-Ile-Asp-Ser-Gln-Glu-Leu-Ser-Lys-
Val-Ser-Arg-Glu-Phe-His-Ser-His-Glu-Phe-
His-Ser-His-Glu-Asp-Met-Leu-Val-Val-Asp-
Pro-Lys-Ser-Lys-Glu-Glu-Asp-Lys-His-Leu-
Lys-Phe-Arg-Ile-Ser-His-Glu-Leu-Asp-Ser-
Ala-Ser-Ser-Glu-Val-Asn

a sequence which is referred to herein as SEQ ID NO 1, as well as proteins having substantial homology thereto, and active peptide portions of the foregoing, are active modulators of mineralization events in mammals, serving as effective inhibitors of calcium oxalate crystal growth. These proteins, which can be isolated from urine, and peptide portions thereof, are useful as therapeutic agents, such as in the treatment of kidney stone disease.

The present invention is also directed to the discovery of extremely active and novel peptide portions contained in the foregoing protein SEQ ID NO 1. One highly active portion is a peptide comprising the sequence

His-Asp-His-Met-Asp-Asp-Met-Asp-Asp-Glu-
Asp-Asp-Asp-Asp-His-Val-Asp-Ser-Gln-Asp-
Ser-Ile-Asp-Ser-Asn-Asp

The present invention is also directed to hybridomas capable of producing monoclonal antibodies to the foregoing proteins and peptides, and to the monoclonal antibodies so produced.

The monoclonal antibodies produced by the hybridomas of the present invention are capable of specifically binding to at least one antigenic determinant of the proteins and peptides. Thus, such monoclonal antibodies find uses, for example, in immunopurification processes for the extraction of the proteins and peptides, and the present invention is further directed to the same. The immunopurification process can be carried out by passing a sample containing proteins or peptides of the invention through an immunoabsorbent column which comprises a monoclonal antibody of the invention bound to a solid phase support.

Such monoclonal antibodies are also useful as diagnostic agents for certain diseases or conditions characterized by an excess or deficiency of proteins or peptides of the invention. Thus, the present invention encompasses immunoassays for detecting the presence of the subject proteins or peptides, such immunoassays comprising contacting fluid of the patient with a monoclonal antibody of the invention and screening for protein-antibody or peptide-antibody interactions (hereinafter referred to collectively as protein-antibody interactions). In addition, the invention contemplates diagnostic kits comprising a protein or peptide of the invention, and a monoclonal antibody to

These and other aspects of the invention will become more apparent from the following detailed description when taken in conjunction with the following figures.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1A shows a DEAE-cellulose chromatogram of gradient salt elution (0.1 M to 0.4 M NaCl) of the DEAE batch eluate obtained from normal human urine partially depleted of TH by salt precipitation. The greatest reactivity of tubes by ELISA (closed circles) using antisera prepared by immunization with the protein fraction corresponding to the main inhibitory peak (identified by functional assay of C^{14} oxalate incorporation into seed crystals) is present in tubes that eluted earlier than the main inhibitory peak (open circles).

Figure 1B shows a DEAE-cellulose chromatogram of gradient salt elution (0.1 M to 0.4 M NaCl) of the DEAE-cellulose batch eluate obtained from normal human urine partially depleted of TH by salt precipitation. The greatest reactivity by ELISA detected with monoclonal antibody ZH2 (closed circles) using microtiter plates coated with column fractions coincided with the protein fraction corresponding to the main inhibitory peak (open circles) which had been identified by functional assay of C^{14} oxalate incorporation into seed crystals.

Figure 1C shows elution of ELISA reactivity of ZH2 (closed circles) and crystal growth inhibitory activity (open circles) from an affinity column of monoclonal antibody ZH2

Figure 4 shows a 16% SDS-PAGE of the uropontin protein of the present invention purified by immunoaffinity chromatography using ZH2 beads. The position of migration of molecular weight markers x 10^3 is shown on the left. Lanes 1 and 2 contain 6 μg samples of uropontin protein per lane and lane 3 contains 6 μg of DEAE batch eluate. Lane 1 was stained with silver. Lanes 2 and 3 are Western blots that used monoclonal antibody ZH2 for detection.

DETAILED DESCRIPTION OF THE INVENTION

The proteins of the present invention include the protein of SEQ ID NO 1 (set forth above). The protein of the SEQ ID NO 1, which was isolated from human urine by immunoaffinity chromotography, has been found by micro-sequencing and amino acid analysis to be homologous to human osteopontin, a protein which is encoded, by the human osteopontin (OPN) gene. Human osteopontin (and the gene encoding that protein) is shown and described in Kiefer et al., *Nucleic Acids Res.*, Vol. 17, pp. 3306-3306 (1989) and Young et al, *Genomics*, Vol. 7, pp. 491-502 (1990), the disclosures of each of which are incorporated herein by reference in their entirety. At times herein, the protein of SEQ ID NO 1 will be referred to as human uropontin or HUP, a name which reflects the fact that it has been isolated from urine.

The present invention is also directed to peptides having substantial homology to the human uropontin protein of SEQ ID NO 1. By the phrase proteins having substantial

Met-Glu-Ser-Glu-Glu-Leu-Asn-Gly-Ala-Tyr-
Lys-Ala-Ile-Pro-Val-Ala-Gln-Asp-Leu-Asn-
Ala-Pro-Ser-Asp-Trp-Asp-Ser-Arg-Gly-Lys-
Asp-Ser-Tyr-Glu-Thr-Ser-Gln-Leu-Asp-Asp-
Gln-Ser-Ala-Glu-Thr-His-Ser-His-Lys-Gln-
Ser-Arg-Leu-Tyr-Lys-Arg-Lys-Ala-Asn-Asp-
Glu-Ser-Asn-Glu-His-Ser-Asp-Val-Ile-Asp-
Ser-Gln-Glu-Leu-Ser-Lys-Val-Ser-Arg-Glu-
Phe-His-Ser-His-Glu-Phe-His-Ser-His-Glu-
Asp-Met-Leu-Val-Val-Asp-Pro-Lys-Ser-Lys-
Glu-Glu-Asp-Lys-His-Leu-Lys-Phe-Arg-Ile-
Ser-His-Glu-Leu-Asp-Ser-Ala-Ser-Ser-Glu-
Val-Asn

such protein being referred to herein as SEQ ID NO 5. Rat osteopontin (and the gene encoding that protein) is shown and described, for example, in Oldberg et al., *Proc. Natl. Acad. Sci. USA*, Vol. 83, pp. 8819-8823 (1986), and is referred to therein as rat osteopontin. Porcine osteopontin (and the gene encoding that protein) is shown and described, for example, in Wrana et al., *Nucleic Acids Res.*, Vol. 17, pp. 10119-10119 (1989), and is referred to therein as porcin osteopontin. Mouse osteopontins (and genes encoding those proteins) are shown and described, for example, in Craig et al., *J. Biol. Chem.*, Vol. 264, pp. 9682-9689 (1989), Miyazaki et al., *Nucleic Acids Res.*, Vol. 17, pp. 3298-3298 (1989), and Patarca et al., *J. Exp. Med.*, Vol. 170, pp. 145-161 (1989), and are referred to therein as 2ar osteopontin, osteopontin and eta-1, respectively. The disclosures of each

about 200 amino acids (inclusive of the fragments SEQ ID NOs 2-4), more preferably no greater than about 150 amino acids, even more preferably no greater than about 100 amino acids, and still more preferably no greater than about 50 amino acids.

The foregoing proteins and peptides may be obtained in various fashions, as will be apparent to those skilled in the art once armed with the present disclosure. For example, the proteins and/or peptides may be isolated from body fluids such as urine or blood using the techniques disclosed herein, may be obtained using standard recombinant DNA techniques such as those described in Sambrook, Fritsch, & Maniatus, *Molecular Cloning: A Laboratory Manual*, Vols. 1-3, 2nd ed. (Cold Springs Harbor Laboratory Press, N.Y. 1989), based on the published gene sequences of the osteopontin proteins, and/or by using conventional peptide synthesis methodology such as is described in Houghton, *Proc. Natl. Acad. Sci. USA*, Vol. 82, pp. 5131-5135 (1985). The disclosures of both of the foregoing publications are incorporated herein by reference in their entirety.

The proteins and peptides of the present invention are useful as therapeutic agents such as in the treatment of kidney stone disease. Accordingly, the present invention contemplates pharmaceutical compositions for treating kidney stone disease comprising an effective amount of the subject proteins or peptides and a pharma-ceutically acceptable carrier and/or diluent. The present invention also contemplates methods for treating kidney stone disease in a

antibodies of the invention may be obtained by the techniques described herein. In general, the subject hybridomas and monoclonal antibodies may be prepared by hyperimmunizing a series of rats (such as Lewis rats) with the proteins or peptides of the invention, obtaining spleen cells (β-lymphocytes) from these rats, and then fusing these spleen cells to myeloma cells (such as Sp2/0-Ag14 myeloma cells) with polyethylene glycol or the like. The resultant hybridoma cells can then be cloned, and antibody-secreting hybridomas can be selected for their ability to react with the proteins or peptides used for immunization. Further details on the procedures for producing hybridomas and monoclonal antibodies is set forth in the examples provided below. In addition, the preparation of hybridomas and monoclonal antibodies is described, for example, in Harlow & Lane, *Antibodies: A Laboratory Manual* (Cold Springs Harbor Laboratory Press, N.Y. 1989), the disclosures of which are incorporated herein by reference in their entirety.

Polyclonal antibodies to the subject proteins and peptides are also within the ambit of the invention. Such polyclonal antibodies may be produced using standard techniques, for example, by immunizing a rabbit (such as a New Zealand rabbit) or a rat with a protein or peptide of the invention, removing serum from the rabbit, and then harvesting the resultant polyclonal antibodies from the serum. If desired, the polyclonal antibodies may be used as an IgG fraction or may be further purified in varying degrees. Procedures for preparing, harvesting and purifying

proteins and peptides include blood, urine, and/or breast milk.

Such monoclonal antibodies and/or polyclonal antibodies are also useful as diagnostic agents for the detection of certain diseases or conditions characterized by an excess or deficiency of the proteins or peptides of the invention. Thus, the present invention encompasses immunoassays for determining the levels of the subject proteins or peptides, such immunoassays comprising contacting fluid of the patient with a monoclonal and/or polyclonal antibody of the invention and screening for protein-antibody interactions. Applications of such diagnostic methods include, for example, the evaluation of patients suspected of having kidney stone disease, a disease where a deficiency in the normal amount of the proteins or peptides in a patient's urine and/or blood indicates the possible existence of such a malady. With respect to the diagnostic utilities decribed herein, the phrase a normal amount of proteins or peptides should be taken to mean that amount of proteins or peptides which would statistically be present in the fluids of normal patient of the same weight and age. Since activated lymphoid tumors and bone tumors secrete an excess amount of the proteins or peptides of the present invention, as compared to normal, the monoclonal and/or polyclonal antibodies of the invention can be used to diagnose the potential magnitude of lymphoid or bone tumors in a patient supected or known to have such tumors by an immunoassay of the urine and/or blood of the patient. Senger et al., *Cancer Research*, Vol. 48, pp.

1942 (1990); Patarca et al., *J. Exp. Med.*, Vol. 170, pp. 145-161 (1989). Employed as described above, the present monoclonal and/or polyclonal antibodies provide a new and important tool for clinical diagnosis and prognosis efforts in the foregoing areas.

The present invention may also be employed diagnostically in immunoassays for determining the relative amounts of proteins or peptides of the invention present in a patient. Such assays may be carried out by contacting blood and/or urine from a patient with a monoclonal and/or polyclonal antibody to one protein or peptide of the invention, such as protein SEQ ID NO 1, and screening for protein-antibody interactions, while concurrently, subsequently, or prior to contacting blood and/or urine from the same patient with a monoclonal and/or polyclonal antibody to another protein or peptide of the invention, such as SEQ ID NO 2, and also screening for protein-antibody interactions. The amount of protein-antibody interactions in the two screenings can then be compared to ascertain the relative amounts of the two proteins or peptides in the patient. This can then be compared against the relative amounts possessed by a normal patient to ascertain whether the patient has an improper balance of proteins or peptides.

The present invention may also be employed diagnostically in immunoassays for monitoring the levels of the proteins or peptides of the invention present in a patient during protein or peptide therapy. Such assays may be carried out by contacting blood and/or urine from a

numerous publications, including *Antibodies: A Laboratory Manual* (Cold Springs Harbor Laboratory Press, N.Y. 1989), the disclosures of which are incorporated herein by reference in their entirety. Conventional diagnostic kit components may include such items as, for example, microtiter plates, buffers (such as, for example, EDTA buffer, Tris buffer, etc.), secondary buffers (such as, for example, peroxidase conjugated anti-rat IgG or anti-rabbit IgG), and other standard reagents and components.

Further, the present invention encompasses the use of the monoclonal antibodies as therapeutic agents, such as in the treatment of osteoporosis. Thus, the present invention contemplates pharmaceutical compositions for treating osteoporosis comprising an effective amount of the subject monoclonal antibodies and a pharmaceutically acceptable carrier and/or diluent. The present invention also contemplates methods for treating osteoporosis, a disease characterized by features suggestive of too much of the subject proteins or peptides as compared to normal, in a patient comprising administering to the patient an effective amount of the subject monoclonal antibodies. The use of the monoclonal antibodies as immunoprophylactic reagents in the treatment of osteoporosis represents a significant new approach in dealing with this disease.

Acceptable carriers and diluents which can be employed in connection with the monoclonal antibodies in the subject pharmaceutical compositions are well known in the pharmaceutical art, and are described, for example, in

minutes, as described in Tamm et al., *Proc. Soc. Exp. Biol. Med.*, Vol. 74, pp. 108-114 (1950). TH-depleted urine was then adsorbed to DEAE-cellulose, batch eluted and fractionated by DEAE-cellulose column chromatography using a 0.1 M to 0.4 M NaCl linear gradient in Tris buffer, as decribed in Nakagawa et al., *J. Biol. Chem.*, Vol. 258, pp. 12594-12600 (1983).

Crystal growth inhibition of the protein fractions was assayed by measuring the inhibition of incorporation of [^{14}C]oxalate (available from Amersham, Arlington Heights, IL) into calcium oxalate monohydrate seed crystals (EM Sciences, Gibbstown, NJ) during an incubation period of 180 minutes, using the method described in Ligabue et al., *Clin. Chim. Acta*, Vol. 98, pp. 39-46 (1979). Samples and standards (200 µl) were incubated with 2.0 ml of a metastable solution (2 mM $CaCl_2$, 0.2 mM sodium oxalate (available from Sigma, St. Louis, MO), 5 mM sodium cacodylate (available from Sigma), 0.15 M NaCl, pH 6.0) and 10 µl of [^{14}C]oxalate (0.1 µCi) for 30 minutes at 37°C in a shaking bath. After equilibration, 200 µl was removed and counted in a liquid scintillation counter (T_0). The remainder was incubated for 180 minutes at 37°C with 200 µl of aged calcium oxalate seed crystal solution (1.5 mg/ml) under constant shaking. One ml samples were then removed and centrifuged (8,000 x g for 2 min at 4°C), and the radioactivity of 200 µl of each supernatant was counted (T_{180}).

The percent residual radioactivity in 200 µl of the centrifuge supernatants was then used to calculate the

or polyclonal antibodies respectively in the buffer for 60 minutes at 37°C, washed, and then incubated with peroxidase conjugated anti-rat IgG or anti-rabbit IgG (available from Cappel, West Chester, PA), respectively, in casein buffer for 60 minutes at 37°C and rewashed. The substrate reaction using a buffer comprising 0.137% O-phenylenediamine (available from Aldrich Chemical Co., Milwaukee, WI), 0.009% H_2O_2 in 0.2 M Tris, and 0.15 M NaCl, at pH 6.0 was performed in the dark at room temperature and the optical density at 490 nM determined in an automated ELISA reader (available from Dynatech). In initial studies of DEAE column fractions using a sandwich ELISA, polyclonal rabbit antibody-coated plates were exposed to dilutions of column fractions and then to polyclonal rat antisera.

SDS-polyacrylamide gel electrophoresis (PAGE) was performed by the slab technique, as generally described in Laemmli, *Nature*, Vol. 227, pp. 68-685 (1970). Specific-ally, SDS-PAGE was performed using samples (6 μg) and molecular weight standards (available from Bio-Rad Laboratories, Rockville Centre, NY) in 2.0% SDS, 0.02 M dithiothreitol (DTT) (available from Bio-Rad Laboratories), and 0.0625 M Tris, at pH 6.8 which were heated for 2 minutes at 90°C. Samples were applied to 16% polyacrylamide (available from FMC Bioproducts, Rockland, ME) or 5 to 18% gradient polyacrylamide slab gels containing 0.375 M Tris, and 0.1% SDS, at pH 8.8. Electrophoresis was performed using a 0.025 M Tris, 0.192 M glycine, 0.1% SDS, pH 8.3 running buffer.

showed no inhibitory activity in functional assays. Examination of the gradient DEAE column fractions by ELISA showed that these antibodies identified ELISA peak reactivity (ELISA peak, EP) in tubes earlier than the inhibitory peak (Figure 1A).

The eluates from these antibody beads migrated in the same position as Tamm-Horsfall protein (TH) on 16% PAGE gels and were detected in Western blots at the same position (~98 kD) by these monoclonal antibodies and by polyclonal antibodies to TH. The presence of TH in inhibitory fractions from DEAE columns was confirmed by Western blotting of PAGE gels. An ELISA analysis of DEAE column chromatography fractions using polyclonal anti-TH showed peak reactivity in a position identical to that shown in Figure 1A. Each of the three monoclonal antibodies reacted strongly with purified human TH by ELISA.

These results were used to design a second series of subclonings using negative selection for TH. This series of subclonings lead to isolation of a functionally active protein by immunoaffinity chromatography. In accordance with the isolation procedure, partial depletion of Tamm-Horsfall protein (TH) by the classical method of salt precipitation and centrifugation was employed as an initial step. Inhibitory fractions isolated after this step were used for subsequent immunizations. Hybridoma cells were initially selected on the basis of greater ELISA reactivity of their supernatants with the protein fraction of Example 1 than with comparable concentrations of TH or the EP shown in Figure 1A.

ELISA with their respective antibodies (Figures 2A and 2B). Inhibitory activity was detected in the eluate from ZH2 beads (Figure 2C).

EXAMPLE 3

Immunoaffinity Purification

Solid-phase immunoabsorbents were prepared by coupling IgG fractions of monoclonal antibodies from Example 2 to cyanogen bromide-activated Sepharose 4B (5 mg protein/ml beads). After exposure to aliquots of inhibitory fractions of human urine, the monoclonal antibody beads were extensively washed with phosphate buffered saline, pH 7.4, followed by elution with a 0.2 M glycine pH 2.8 buffer. Eluates were neutralized and dialysed against a 0.05 M Tris, 0.05 M NaCl, pH 7.3 buffer prior to characterization of inhibitory activity and immunologic reactivity. The protein isolated by immunoaffinity chromatography was further purified by reverse-phase HPLC, using the procedures described in Przysiecki et al, *Proc. Natl. Acad. Sci. USA*, Vol. 84, pp. 7858-7860 (1987), prior to analysis of amino acid composition and N-terminal sequence.

Affinity columns of ZH2 beads were used for purification of an inhibitory protein based on the results in Example 2. The elution patterns for inhibitory activity and ELISA reactivity with ZH2 of these large columns were substantially the same as those shown in Figure 1B. Approximately 30% of the protein in 3-9 liter lots of TH-depleted urine (n = 4) was isolated by 0.4 M NaCl elution after batch adsorption to DEAE-cellulose. Approximately 4%

Biochem. Biophys. Acta, Vol. 996, pp. 43-48 (1989)). The amino acid sequences of the four osteopontins shown start with position 19 of the precursors and extend to residue 62 (residue 61 for MOP). With the exception of an indeterminate residue 25, the entire N-terminal sequence from residue 2 to 30 of HUP isolated from a second individual was identical to that shown. The last 5 of the 7 amino acids in the N-terminus of human lactopontin are identical to the first 5 amino acids of HUP. The last 4 amino acids (Asn-Ala-Val-Ser) of the HUP sequence are deleted in one of the isoforms encoded by mRNA from human bone, decidua, Young et al., *Genomics*, Vol. 7, pp. 491-502 (1990), and kidney.

The amino acid composition determined for human uropontin, shown in Table 1, includes a very high percentage of aspartic acid residues and corresponds to the distribution of amino acids in human osteopontin. The composition was determined using standard acid hydrolysis and chromatographic techniques, as decribed in Fisher et al., *J. Biol. Chem.*, Vol. 262, pp. 9702-9708 (1987) and Przysiecki et al., *Proc. Natl. Acad. Sci. USA*, Vol. 84, pp. 7858-7860 (1987). Uropontin, however, is quite distinct from nephrocalcin, another protein inhibitor of crystal growth. Nakagawa et al., *J. Biol. Chem.*, Vol. 258, pp. 12594-12600 (1983). The amino acid composition and molecular weights of the two proteins differ substantially, and none of the glutamic acid residues in uropontin are γ-carboxylated as they are in nephrocalcin, osteocalcin and other vitamin K-dependent proteins. The amino acid and nucleotide sequences of

EXAMPLE 5

Gene Cloning

To clone the gene encoding the uropontin protein of the invention, an adult human kidney λ gt10 cDNA library (purchased from Clontech, in California) was screened using two oligonucleotide probes having the following nucleotide sequences.

Probe 1 5'-CTGATTCTGGAAGTTCTGAGGA-3'

Probe 2 5'-AGATTCTGCTTCTGAGATGGGTCAGG-3'

Probe 1 corresponds to nucleotides 132-153 of the nucleotide sequence of the osteopontin gene, as published in Kiefer et al., *Nucleic Acids Res.*, Vol. 17, pp. 3306-3308 (1989) (hereinafter referred to as "Kiefer"), and to amino acids 4-11 (from the N-terminus) of SEQ ID NO 1 shown herein. Probe 2 corresponds to nucleotides 203-228 of the osteopontin gene published in Kiefer, and to amino acids 28-36 (from the N-terminus) of SEQ ID NO 1 shown herein. About 250,000 clones were initially screened with ^{32}P-labeled oligonucleotide and 40 positives were identified. Three of these positives were subcloned, the DNA of these clones was amplified by PCR using λ gt10 forward and reverse primers and ligated into pUC19, and DNA minipreps containing inserts were sequenced using the dideoxy method and ^{35}S-dATP. To complete the sequencing, two additional oligonucleotide primers having the following sequences were employed.

Primer 1 5'-GAAAGCCATGACCACATGGA-3'

Primer 2 5'-TTGACCTCAGAAGATG(A,C)ACT-3'

TABLE 1

Amino Acid Composition (Residues/1000)

Amino Acid	Uropontin*	Osteopontin +	Osteopontin ±	Nephrocalcin§
CYSTINE	0	ND¶	0	17
ASN+ASP	207	223	201	106
MET	7	ND	13	6
THR	66	40	47	88
SER	162	122	141	97
GLU+GLN	140	176	138	122
PRO	55	76	50	59
GLY	69	25	20	108
ALA	52	55	47	76
VAL	46	42	60	64
ILE	15	23	23	24
LEU	47	60	54	63
TYR	26	9	27	10
PHE	16	8	23	31
HIS	36	46	54	20
LYS	39	64	64	36
ARG	17	28	30	41
TRYP	ND	ND	7	9
GLA#	0	ND	ND	20

* uropontin isolated from human urine.

\+ osteopontin isolated from human bone.

± human osteopontin predicted by cDNA for a mature protein sequence of 298 amino acids. This sequence predicts that 164 of the 201 (Asp + Asn) residues/1000 are aspartic acid and that 103 of the 138 (Glu + Gln) residues/1000 are glutamic acid (12)

§ nephrocalcin isolated from human urine.

¶ ND - not done.

\# γ-carboxyglutamic acid. In contrast to uropontin, other proteins also present in the main inhibitory peak contained both GLA and β-hydroxyasparagine.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Hoyer et al.

(ii) TITLE OF INVENTION: Biological Regulation of Mineralization

(iii) NUMBER OF SEQUENCES: 5

(iv) CORRESPONDENCE ADDRESS:

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(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: DISKETTE, 3.5 INCH, 1.44 Mb STORAGE

(B) COMPUTER: IBM PS/2

(C) OPERATING SYSTEM: PC-DOS

(D) SOFTWARE: WORDPERFECT 5.0

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(B) FILING DATE: herewith

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Suzanne E. Miller

125 130 135

Asp-Thr-Tyr-Asp-Gly-Arg-Gly-Asp-Ser-Val-Val-Tyr-Gly-Leu-Arg-

140 145 150

Ser-Lys-Ser-Lys-Lys-Phe-Arg-Arg-Pro-Asp-Ile-Gln-Tyr-Pro-Asp-

155 160 165

Ala-Thr-Asp-Glu-Asp-Ile-Thr-Ser-His-Met-Glu-Ser-Glu-Glu-Leu-

170 175 180

Asn-Gly-Ala-Tyr-Lys-Ala-Ile-Pro-Val-Ala-Gln-Asp-Leu-Asn-Ala-

185 190 195

Pro-Ser-Asp-Trp-Asp-Ser-Arg-Gly-Lys-Asp-Ser-Tyr-Glu-Thr-Ser-

200 205 210

Gln-Leu-Asp-Asp-Gln-Ser-Ala-Glu-Thr-His-Ser-His-Lys-Gln-Ser-

215 220 225

Arg-Leu-Tyr-Lys-Arg-Lys-Ala-Asn-Asp-Glu-Ser-Asn-Glu-His-Ser-

230 235 240

Asp-Val-Ile-Asp-Ser-Gln-Glu-Leu-Ser-Lys-Val-Ser-Arg-Glu-Phe-

245 250 255

His-Ser-His-Glu-Phe-His-Ser-His-Glu-Asp-Met-Leu-Val-Val-Asp-

260 265 270

Pro-Lys-Ser-Lys-Glu-Glu-Asp-Lys-His-Leu-Lys-Phe-Arg-Ile-Ser-

275 280 285

His-Glu-Leu-Asp-Ser-Ala-Ser-Ser-Glu-Val-Asn

290 295

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 amino acids

(B) TYPE: amino acid

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 282 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Val-Lys-Gln-Ala-Asp-Ser-Gly-Ser-Ser-Glu-Glu-Lys-Gln-Leu-Tyr-
1 5 10 15

Asn-Lys-Tyr-Pro-Asp-Ala-Val-Ala-Thr-Trp-Leu-Asn-Pro-Asp-Pro-
20 25 30

Ser-Gln-Lys-Gln-Asn-Leu-Leu-Ala-Pro-Gln-Thr-Leu-Pro-Ser-Lys-
35 40 45

Ser-Asn-Glu-Ser-His-Asp-His-Met-Asp-Asp-Met-Asp-Asp-Glu-Asp-
50 55 60

Asp-Asp-Asp-His-Val-Asp-Ser-Gln-Asp-Ser-Ile-Asp-Ser-Asn-Asp-
65 70 75

Ser-Asp-Asp-Val-Asp-Asp-Thr-Asp-Asp-Ser-His-Gln-Ser-Asp-Glu-
80 85 90

Ser-His-His-Ser-Asp-Glu-Ser-Asp-Glu-Leu-Val-Thr-Asp-Phe-Pro-
95 100 105

Thr-Asp-Leu-Pro-Ala-Thr-Glu-Val-Phe-Thr-Pro-Val-Val-Pro-Thr-
110 115 120

Val-Asp-Thr-Tyr-Asp-Gly-Arg-Gly-Asp-Ser-Val-Val-Tyr-Gly-Leu-
125 130 135

Arg-Ser-Lys-Ser-Lys-Lys-Phe-Arg-Arg-Pro-Asp-Ile-Gln-Tyr-Pro-
140 145 150

Asp-Ala-Thr-Asp-Glu-Asp-Ile-Thr-Ser-His-Met-Glu-Ser-Glu-Glu-
155 160 165

CLAIMS

What is claimed is:

1. A composition for treating kidney stone disease comprising an effective amount of a protein comprising the protein of SEQ ID NO 1, a protein having substantial homology thereto, or active portions thereof, in combination with a pharmaceutically acceptable carrier or diluent.

2. A composition of Claim 1 wherein the protein is the protein of SEQ ID NO 1.

3. A composition of Claim 1 wherein the protein is a protein having substantial homology to SEQ ID NO 1 which is the protein of SEQ ID NO 5.

4. A method for treating kidney stone disease in a patient comprising administering to the patient an effective amount of a composition of Claim 1.

5. A method for treating kidney stone disease in a patient comprising administering to the patient an effective amount of a composition of Claim 2.

6. A method for treating kidney stone disease in a patient comprising administering to the patient an effective amount of a composition of Claim 3.

7. A hybridoma capable of producing monoclonal antibody to a protein comprising protein SEQ ID NO 1, a protein having substantial homology thereto, or active portions thereof.

8. A hybridoma of Claim 7 wherein the protein is SEQ ID NO 1.

9. A hybridoma of Claim 7 wherein the protein is a protein having substantial homology to SEQ ID NO 1 which is SEQ ID NO 5.

10. A monoclonal antibody wherein the antibody is capable of specifically binding to at least one antigenic determinant of a protein comprising the protein of SEQ ID NO 1, a protein having substantial homology thereto, or active portions thereof.

(i) contacting blood or urine from the patient with a monoclonal antibody of Claim 11; and

(ii) screening for protein-antibody interactions.

21. An immunoassay for diagnosing kidney stone disease in a patient, comprising:

(i) contacting blood or urine from the patient with a monoclonal antibody of Claim 12; and

(ii) screening for protein-antibody interactions.

22. An immunoassay for diagnosing the magnitude of lymphoid or bone tumors in a patient, comprising:

(i) contacting blood or urine from the patient with a monoclonal antibody of Claim 10; and

(ii) screening for protein-antibody interactions.

23. An immunoassay for diagnosing the magnitude of lymphoid or bone tumors in a patient, comprising:

(i) contacting blood or urine from the patient with a monoclonal antibody of Claim 11; and

(ii) screening for protein-antibody interactions.

24. An immunoassay for diagnosing the magnitude of lymphoid or bone tumors in a patient, comprising:

(i) contacting blood or urine from the patient with a monoclonal antibody of Claim 12; and

(ii) screening for protein-antibody interactions.

25. An immunoassay for diagnosing osteoporosis in a patient, comprising:

30. An immunoassay for diagnosing the susceptability of a patient to infections by obligatory intracellular organisms comprising:

(i) contacting blood or urine from the patient with a monoclonal antibody of Claim 12; and

(ii) screening for protein-antibody interactions.

31. An immunoassay for diagnosing autoimmune disease in a patient comprising:

(i) contacting blood or urine from the patient with a monoclonal antibody of Claim 10; and

(ii) screening for protein-antibody interactions.

32. An immunoassay for diagnosing autoimmune disease in a patient comprising:

(i) contacting blood or urine from the patient with a monoclonal antibody of Claim 11; and

(ii) screening for protein-antibody interactions.

33. An immunoassay for diagnosing autoimmune disease in a patient comprising:

(i) contacting blood or urine from the patient with a monoclonal antibody of Claim 12; and

(ii) screening for protein-antibody interactions.

34. A method for treating osteoporosis in a patient comprising administering to the patient an effective amount of a monoclonal antibody of Claim 10.

35. A method for treating osteoporosis in a patient comprising administering to the patient an effective amount of a monoclonal antibody of Claim 11.

44. A peptide of Claim 43 wherein said total number of amino acids in the peptide is less than about 50.

45. A peptide of Claim 44 which is the peptide of SEQ ID NO 2.

46. A composition for treating kidney stone disease comprising an effective amount of a peptide of Claim 41 in combination with a pharmaceutically acceptable carrier or diluent.

47. A composition for treating kidney stone disease comprising an effective amount of a peptide of Claim 45 in combination with a pharmaceutically acceptable carrier or diluent.

48. A method for treating kidney stone disease in a patient comprising administering to the patient an effective amount of a composition of Claim 46.

49. A method for treating kidney stone disease in a patient comprising administering to the patient an effective amount of a composition of Claim 47.

50. A hybridoma capable of producing monoclonal antibody to a peptide comprising the peptide of SEQ ID NO 2, or a peptide sequence having substantial homology thereto.

51. A hybridoma of Claim 50 wherein the peptide is the peptide of SEQ ID NO 2.

52. A monoclonal or polyclonal antibody wherein the antibody is capable of specifically binding to at least one antigenic determinant of a peptide comprising the peptide of SEQ ID NO 2, or a peptide sequence having substantial homology thereto.

53. A monoclonal or polyclonal antibody of Claim 52 wherein the peptide is the peptide of SEQ ID NO 2.

54. A monoclonal antibody produced from a hybridoma of Claim 50.

55. A monoclonal antibody produced from a hybridoma of Claim 51.

56. An immunopurification process for extracting a peptide comprising the peptide of SEQ ID NO 2, or a

(ii) screening for protein-antibody interactions.

62. A method for treating osteoporosis in a patient comprising administering to the patient an effective amount of a monoclonal antibody of Claim 52.

63. A method for treating osteoporosis in a patient comprising administering to the patient an effective amount of a monoclonal antibody of Claim 53.

64. A diagnostic kit comprising a peptide comprising the peptide of SEQ ID NO 2, or a peptide having substantial homology thereto, and a monoclonal or polyclonal antibody to that peptide, in combination with conventional diagnostic kit components.

65. A diagnostic kit of Claim 64 wherein the peptide is the peptide of SEQ ID NO 2.

66. A peptide comprising the peptide of SEQ ID NO 3, or a peptide sequence having substantial homology thereto, provided that the total number of amino acids in the peptide is less than about 200.

67. A peptide of Claim 66 wherein said total number of amino acids in the peptide is less than about 150.

68. A peptide of Claim 67 wherein said total number of amino acids in the peptide is less than about 100.

69. A peptide of Claim 68 wherein said total number of amino acids in the peptide is less than about 50.

70. A peptide of Claim 69 which is SEQ ID NO 3.

71. A composition for treating kidney stone disease comprising an effective amount of a peptide of Claim 66 in combination with a pharmaceutically acceptable carrier or diluent.

72. A composition for treating kidney stone disease comprising an effective amount of a peptide of Claim 70 in combination with a pharmaceutically acceptable carrier or diluent.

83. An immunoassay for diagnosing kidney stone disease in a patient, comprising:

(i) contacting blood or urine from the patient with a monoclonal or polyclonal antibody of Claim 77; and

(ii) screening for protein-antibody interactions.

84. An immunoassay for diagnosing kidney stone disease in a patient, comprising:

(i) contacting blood or urine from the patient with a monoclonal or polyclonal antibody of Claim 78; and

(ii) screening for protein-antibody interactions.

85. An immunoassay for diagnosing osteoporosis in a patient, comprising:

(i) contacting blood or urine from the patient with a monoclonal or polyclonal antibody of Claim 77; and

(ii) screening for protein-antibody interactions.

86. An immunoassay for diagnosing osteoporosis in a patient, comprising:

(i) contacting blood or urine from the patient with a monoclonal or polyclonal antibody of Claim 78; and

(ii) screening for protein-antibody interactions.

87. A method for treating osteoporosis in a patient comprising administering to the patient an effective amount of a monoclonal antibody of Claim 77.

88. A method for treating osteoporosis in a patient comprising administering to the patient an effective amount of a monoclonal antibody of Claim 78.

89. A diagnostic kit comprising a peptide comprising the peptide of SEQ ID NO 3, or a peptide having substantial homology thereto, and a monoclonal antibody or

101. A hybridoma of Claim 100 wherein the peptide is the peptide of SEQ ID NO 4.

102. A monoclonal or polyclonal antibody wherein the antibody is capable of specifically binding to at least one antigenic determinant of a peptide comprising the peptide of SEQ ID NO 4, or a peptide sequence having substantial homology thereto.

103. A monoclonal or polyclonal antibody of Claim 102 wherein the peptide is the peptide of SEQ ID NO 4.

104. A monoclonal antibody produced from a hybridoma of Claim 100.

105. A monoclonal antibody produced from a hybridoma of Claim 101.

106. An immunopurification process for extracting a peptide comprising the peptide of SEQ ID NO 4, or a peptide sequence having substantial homology thereto, from a sample containing said peptide, wherein said sample is passed through an immunoabsorbent column comprising a monoclonal or polyclonal antibody of Claim 102 bound to a solid phase support.

107. An immunopurification process for extracting a peptide which is peptide SEQ ID NO 4, from a sample containing said peptide, wherein said sample is passed through an immunoabsorbent column comprising a monoclonal or polyclonal antibody of Claim 103 bound to a solid phase support.

108. An immunoassay for diagnosing kidney stone disease in a patient, comprising:

(i) contacting blood or urine from the patient with a monoclonal or polyclonal antibody of Claim 102; and

(ii) screening for protein-antibody interactions.

109. An immunoassay for diagnosing kidney stone disease in a patient, comprising:

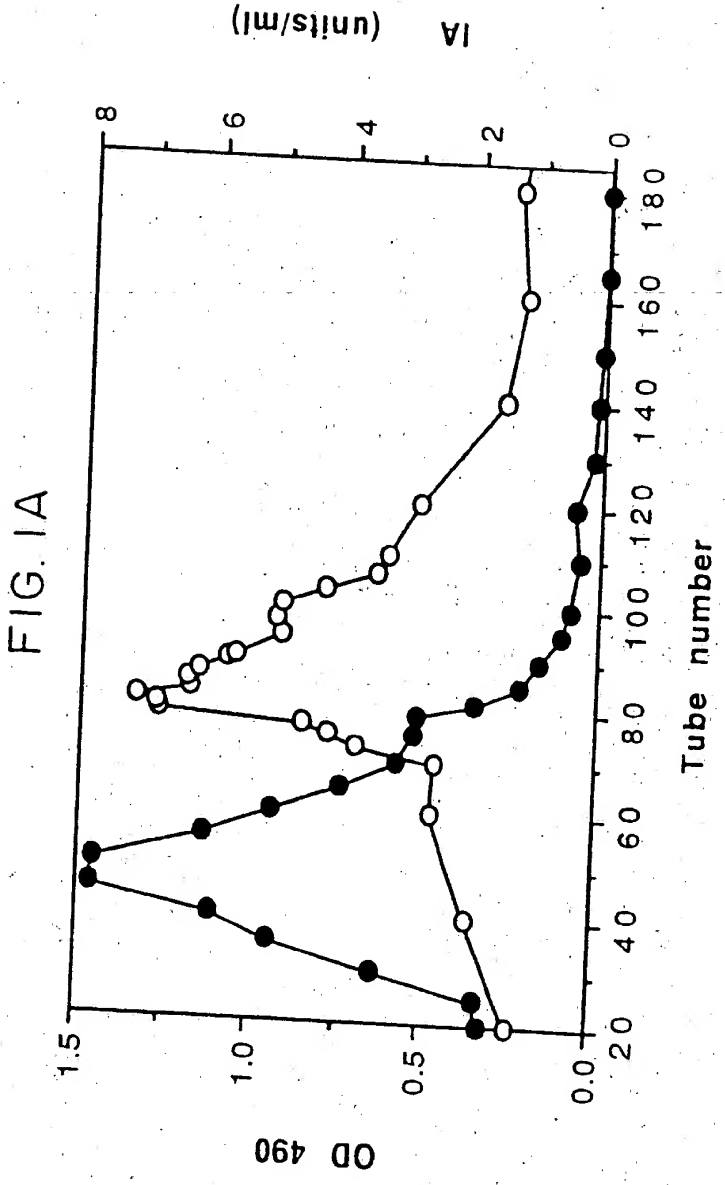


FIG. IA

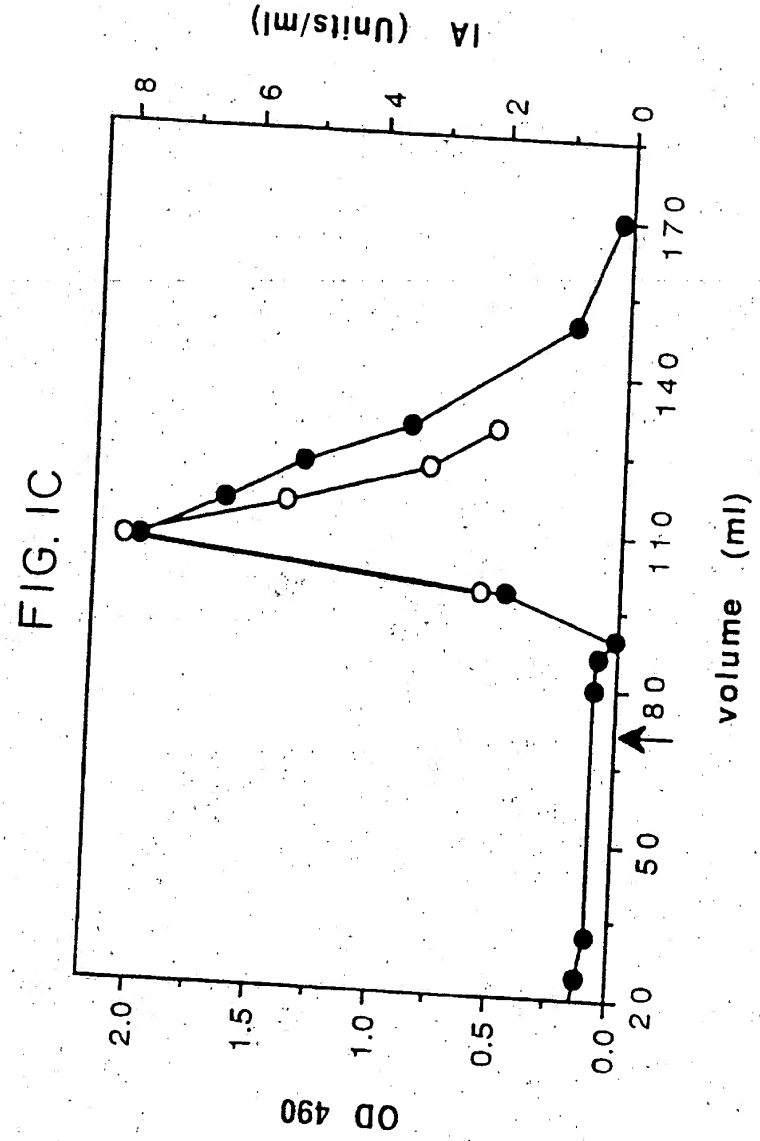
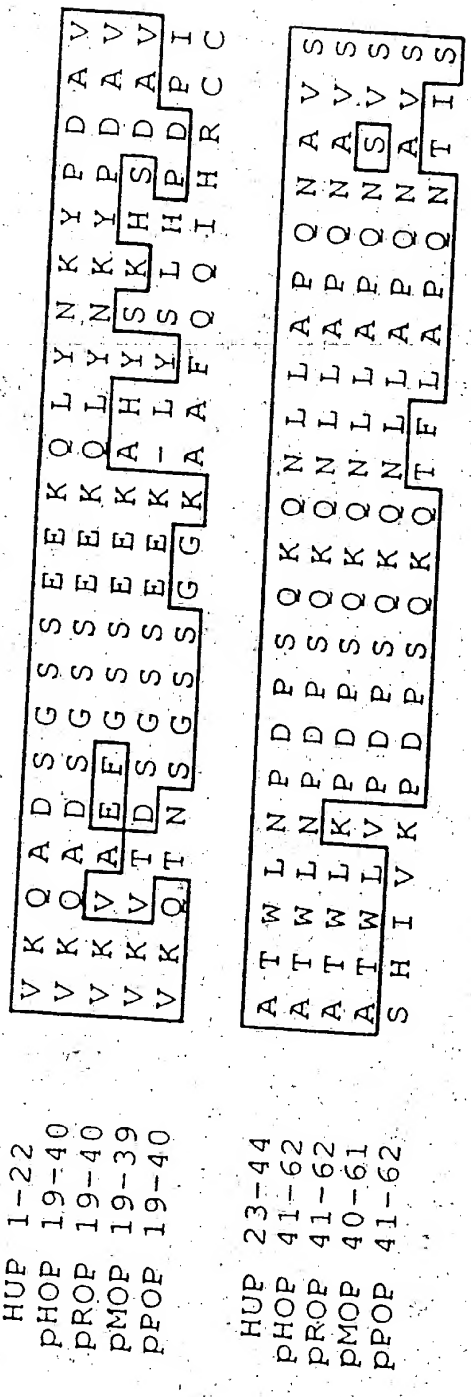


FIG. IC

FIG. 3


HUP 1-22
pHOP 19-40
pROP 19-40
pMOP 19-39
pPOP 19-40
HUP 23-44
pHOP 41-62
pROP 41-62
pMOP 40-61
pPOP 41-62

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/04599

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 37/02, 39/395; C12N 5/12; C07K 15/28,3/20,13/00; G01N 33/68
US CL :424/85.8; 435/240.27; 436/86; 514/8, 12, 14; 530/350, 387.9, 413
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/85.8; 435/240.27; 436/86; 514/8, 12, 14; 530/350, 387.9, 413

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS ONLINE, MEDLINE, BIOSIS,
search terms: uropontin, osteopontin, crystal growth inhibitor, urine

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P Y	US, A, 5,049,659 (Cantor et al) 17 September 1991, particularly Figure 2 and Examples 5.1-5.4, 5.6, 5.8 and 6.2-6.6.	7-15, 19-33 1-3, 16-18, 37-39
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCE, Volume 83, issued December 1986, A. Oldberg et al., "Cloning and Sequence Analysis of Rat Bone Sialoprpotein (Osteopontin) cDNA Reveals an Arg-Gly-Asp Cell Binding Sequence", pages 8819-8823, particularly paragraph 3, page 8819.	4-6
Y	US, A, 4,828,821 (Kelley) 09 May 1989, particularly Figure 7 and Example 4.	4-6
Y	BICHIMICA ET BIOPHYSICA ACTA, Volume 996, issued 1989, D.R. Senger et al., "Purification of a Human Milk Protein Closely Similar to Tumor-Secreted Phosphoproteins and Osteopontin", pages 43-48, particularly the introduction and Table IV.	22-24
Y	JOURNAL OF EXPERIMENTAL MEDICINE, volume 170, issued July 1989, R. Patarca et al., "Structural and Functional Studies of the Early T Lymphocyte Activation 1 (Eta-1) Gene", pages 145-161, entire document.	28-30

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
08 September 1992	15 SEP 1992
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer REBECCA PROUTY Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*